SD - β-Galactosidase - [illegible]

J. Pharm. Pharmacol. 1994, 46: 458–467
Received March 22, 1993
Accepted August 26, 1993

The Effect of Process and Formulation Variables on the Properties of Spray-dried β-Galactosidase

J. BROADHEAD, S. K. EDMOND ROUAN*, I. HAU* AND C. T. RHODES

*Department of Pharmaceutics, University of Rhode Island, Kingston, RI 02881, USA, and *SmithKline Beecham Pharmaceuticals, 709 Swedeland Road, King of Prussia, PA 19406, USA*

Abstract—The objective of this study was to evaluate the joint effects of various processing and formulation variables on the properties of spray-dried β-galactosidase using statistically designed experiments. The key response variables evaluated were product yield, residual enzymatic activity, moisture content and particle size and appearance. The residual enzymatic activity and product yield were significantly affected by the processing variables. The highest product yields were obtained when the drier outlet temperature was relatively high, resulting in extensive protein denaturation. Subsequent experiments, therefore, compared the relative effectiveness of four stabilizers (mannitol, sucrose, arginine hydrochloride and trehalose) in terms of their ability to preserve enzymatic activity during the spray-drying process and during long-term storage. Trehalose was the most suitable stabilizer. The effect of a number of other formulation variables (total solids level, ratio of stabilizer to protein, presence of surfactant and presence of buffer) was also investigated. A final formulation consisting of 6% β-galactosidase and 10% trehalose in deionized water was selected. Spray-drying at inlet and outlet temperatures of 140 and 95°C, respectively, results in greater than 70% yields of a fully active product with a moisture content of 2–5% and a mean particle size of 2–4 μm.

Spray-drying has been used extensively in the pharmaceutical industry, primarily in the production of raw drug materials (such as antibiotics) and excipients (spray-dried lactose). Other well established pharmaceutical applications include granulation and microencapsulation processes (Broadhead et al 1992). Interest in developing novel delivery systems for protein and peptide drugs has focussed attention on spray-drying as a means of processing these thermolabile materials. The potential of spray-drying in protein formulations lies in its ability to produce particles of controlled size and shape. This is critical for the development of systems such as dry powders suitable for inhalation and microspheres for controlled drug delivery.

Spray-drying is already used extensively for drying other heat sensitive materials including enzymes, blood products and microorganisms (Masters 1985). In spite of the high temperatures which are involved, the cooling effect caused by solvent evaporation means that the temperature of the dried product remains relatively low (Masters 1990). Therefore, biologically active materials can be spray-dried without activity losses occurring. [illegible] microspheres using spray-drying techniques highlights the potential of spray-drying for the processing of proteins [illegible] 1980). It has also been reported recently that formulations of tissue plasminogen activator and human growth hormone can be spray-dried at temperatures of 150°C with little or no activity loss (Mumenthaler et al 1991).

Despite the potential advantages of spray-drying, there are a number of difficulties which must be overcome. A major problem often encountered with laboratory-scale spray-driers is a low product recovery. This may be prohibitive in the early stages of pharmaceutical development when only small quantities of very costly drugs are available. In addition, spray-drying is usually associated with product moisture contents considerably higher than those which have been established for marketed lyophilized products.

The aim of this study was, therefore, to evaluate the effects of process and formulation parameters on the characteristics of a model spray-dried protein. The enzyme β-galactosidase, derived from *Aspergillus oryzae*, was chosen as a model. This is a monomeric protein of molecular weight 105 kDa, with an isoelectric point of around pH 4·5 (Tanaka et al 1975; Ogushi et al 1980). Statistically designed experiments were used throughout this study so that the effects of several process and formulation variables could be evaluated simultaneously. The study was carried out in two stages. In the first stage, the goal was to study how process variables (temperature, solution feed rate and air flow rate) affected product characteristics (responses). The second stage of the study focused on the use of formulation excipients to preserve enzymatic activity under conditions where the highest yields could be obtained. The effects of excipient, total solids and the inclusion of a surfactant or buffer salts in the formulation were evaluated, and a final formulation and process established.

Materials and Methods

Materials

Lyophilized β-galactosidase derived from *Aspergillus oryzae* was purchased from Enzyme Development Corporation (New York, USA). The reagents and standard used for the BCA protein assay were purchased from Pierce Chemical Company (Rockford, IL). Tween 80 was purchased from ICI Chemicals (New Brunswick, NJ). Mannitol, O-nitrophenyl-β-D-galactopyranoside (ONPG), sucrose, trehalose, arginine hydrochloride and all other chemicals were purchased from Sigma Chemical Company.

Feed solution preparation

A dispersion of the commercially obtained β-galactosidase

Correspondence: J. Broadhead, Department of Pharmaceutics, University of Rhode Island, Kingston, RI 02881, USA.

was prepared in deionized water (10 g/60 mL). This was centrifuged at 12 000 rev min^{-1} for 15 min to remove the insoluble material present in the commercial preparation. The supernatant was then dialysed against deionized water for 24 h with three changes. This process resulted in a final protein concentration of approximately 60 mg mL^{-1}. The protein solution was diluted to the appropriate concentration with deionized water, when required, and any formulation excipients were added before spray-drying.

Spray-drying

The spray-drying was carried out using a Büchi 190 mini spray-drier. This is a co-current two-fluid drier in which the atomizing air and feed solution pass separately to the nozzle. The feed is atomized by the air as it leaves the nozzle and enters the top of the drying chamber. The dried product is separated from the air stream by means of a cyclone separator. The process variables which can be altered are the drier inlet temperature, the solution feed rate, the air flow rate and the aspirator vacuum. The outlet temperature cannot be controlled directly, but is a function of the drier inlet temperature and the solution feed rate. The aspirator vacuum was kept at approximately 35 mbar throughout the studies. The yield was calculated as the percentage of the solids present in the feed solution which were recovered in the product collector.

Activity determination

The activity of the spray-dried β-galactosidase was determined using a modified method of the Food and Chemical Codex (1981). Approximately 1 mg mL^{-1} solutions of the spray-dried protein were prepared in 0·1 M acetate buffer, pH 4·5. Fifty and 100 μL portions of these solutions were added to 3 mL of ONPG substrate solution (493 mg/100 mL buffer) in a 3·5 mL cuvette. The activity of the samples was calculated by measuring the rate of increase in absorbance at 420 nm caused by the enzyme-catalysed hydrolysis of ONPG to *o*-nitrophenol. A Perkin Elmer Lumbda 7 UV-vis spectrophotometer, maintained at 37°C by a circulating water bath, was used for the activity determinations.

The protein content of the samples was determined using the BCA method (Smith et al 1985) which quantitatively measures protein by the enhanced colorimetric detection of Cu^{+} produced in the reaction of protein with alkaline Cu^{2+}. Bovine serum albumin (BSA) was used as a protein standard. Since some spray-dried samples contained fragments which were insoluble in buffer, the total protein content of the samples was determined after solubilization in 8 M urea. The fraction of insoluble protein was calculated by comparing the protein measurements in the presence and absence of urea. The specific activity of the spray-dried samples (activity (mg of total protein)$^{-1}$) could thus be calculated. Residual activity was expressed as a percentage of the specific activity of the original lyophilized material.

Moisture content determination

The moisture content of the spray-dried samples was determined using a Mitsubishi Moisture Meter (model CA-06, with vaporizer VA-06), in which the samples were heated to 120°C for 10 min before Karl Fischer titration (Johnson 1967).

Particle size analysis

The particle size of the samples was determined using image analysis (Magiscan, Joyce Loebl). Fifty milligram samples of spray-dried material were dispersed in 15 mL silicone fluid and sonicated for 1 min. Four slides were prepared from each dispersion and four fields of view were analysed from each slide. Approximately 400–600 particles from each sample were measured. The Feret diameter (horizontally) was used to represent the sizes of the particles. The geometric mean size was used as the basis for comparing different samples since the size distributions appeared to follow a log-normal pattern.

Scanning electron micrographs (SEM)

Samples of the spray-dried powders were sprinkled on SEM stubs and gold coated. Scanning electron micrographs were obtained using an Amray 1200C Instrument.

Experimental design

Initially, a model formulation consisting of 6% β-galactosidase and 5% mannitol in deionized water was used to evaluate the effect of process variables on the properties of the product. A 2^3 full factorial experimental design was utilized (Box et al 1978). In such a design, three factors are evaluated, each at two levels, and experimental runs are carried out at all eight possible combinations of the levels. Preliminary experiments were carried out to establish appropriate ranges for the processing variables. The high and low temperatures were selected such that activity losses would be expected at the high but not at the low temperature. The high level for the feed rate of 5 mL min^{-1} was the maximum rate which could be used at the low temperature without condensation appearing in the drying chamber, whereas the low level of 2 mL min^{-1} was the slowest practical rate. The high air flow rate was the maximum which was easily achievable using the Büchi 190 drier, whilst the low level of 500 L h^{-1} was the minimum required to provide sufficient energy for atomization. Two replicates of each experimental run were carried out to estimate the inherent variability of the experiments (both measurement variability and other random errors in the system). Since all the runs and duplicates could not be completed in one day, the sixteen runs were carried out in two sets one week apart. So that between-day variability could be estimated and separated from the process variable effects, the sixteen runs were designed in two blocks. The blocking variable was designed in such a way that it was only confounded with the three factor interaction effect, which is considered to be of least interest (Box et al 1978). The experimental design is shown in Table 1.

A 2^5 full factorial experimental design was subsequently used to examine the effect of formulation variables on the spray-dried product (Table 2). This study examined the effect of stabilizer (arginine hydrochloride vs trehalose), surfactant (0·05% Tween 80), total solids concentration, ratio of stabilizer to enzyme, and drying temperature, on the final product. This five-factor design required 32 runs. The experiments were blocked into four sets, carried out at weekly intervals. Duplicate runs were not carried out, since the previous study had not indicated substantial experimental variability. Computations were performed using programs in SAS (Statistical Application Software).

Table 1. Experimental design used to evaluate the effect of processing variables.

Run	Inlet temperature X_1	Feed rate X_2	Airflow rate X_3	Day X_4
1	−1	−1	−1	1
2	+1	−1	−1	2
3	−1	+1	−1	2
4	+1	+1	−1	1
5	−1	−1	+1	2
6	+1	−1	+1	1
7	−1	+1	+1	1
8	+1	+1	+1	2
9	0	0	0	2

Inlet temperature, +140°C; −70°C. Pumping speed: +5 mL min^{-1}; −2 mL min^{-1}. Airflow rate: +700 L h^{-1}; −500 L h^{-1}.

Table 2. Experimental design used to evaluate the effect of formulation variables.

	X_1	X_2	X_3	X_4	X_5	
Week 1						
1	−	−	−	+	+	X_1: surfactant
2	+	−	+	−	+	+present
3	+	−	+	+	−	−absent
4	+	+	0	0	0	
5	−	−	−	−	−	X_2: stabilizer
6	+	+	−	−	−	+trehalose
7	+	+	−	+	+	−arginine HCl
8	−	+	+	−	+	
9	−	+	+	+	−	X_3: total solids
Week 2						+16%
1	−	−	+	−	−	−8%
2	−	+	−	+	−	X_4: stabilizer:enzyme ratio
3	+	+	+	−	−	
4	+	−	−	−	+	+7:1
5	+	−	−	+	−	−1·67:1
6	−	+	−	−	+	
7	+	+	+	+	+	X_5: inlet temperature
8	−	−	0	0	0	+175°C
9	−	−	+	+	+	−105°C
Week 3						
1	−	−	+	−	+	
2	−	+	−	+	+	X_6: $X_1 \cdot X_3 \cdot X_5$
3	+	−	−	−	−	X_7: $X_1 \cdot X_4 \cdot X_5$
4	+	+	+	+	−	
5	−	−	+	+	−	
6	+	−	−	+	+	
7	−	+	−	−	−	
8	−	+	0	0	0	
9	+	+	+	−	+	
Week 4						
1	+	−	0	0	0	
2	+	−	+	−	−	
3	+	+	−		+	
4	−	+	+	−	−	
5	+	−	+	+	+	
6	−	+	+	+	+	
7	+	+	−	+	−	
8	−	−	−	−	+	
9	−	−	−	+	−	

	X_6	X_7
Week 1	−	−
Week 2	+	+
Week 3	+	−
Week 4	−	+

Results and Discussion

Activity losses induced by spray-drying

Initial experiments were carried out in which β-galactosidase was spray-dried in the absence of any protective excipient. Fig. 1 shows the effect of drying temperature (inlet and outlet) on the residual enzymatic activity. It is evident that even at the lowest possible drying temperatures, activity losses occur. Fig. 2 shows the relationship between the residual activity of the above samples, and the presence of insoluble protein. The formation of insoluble protein aggregates is clearly involved in the observed activity losses, although this phenomenon only occurs with activity losses greater than about 15%. Izutsu et al (1991) observed that when freeze-dried β-galactosidase (derived from *A. oryzae*) was stored at 70°C, soluble higher molecular weight aggregates formed which were associated with activity losses. This may also occur as a result of spray-drying, with insoluble precipitates forming when denaturation is more extensive.

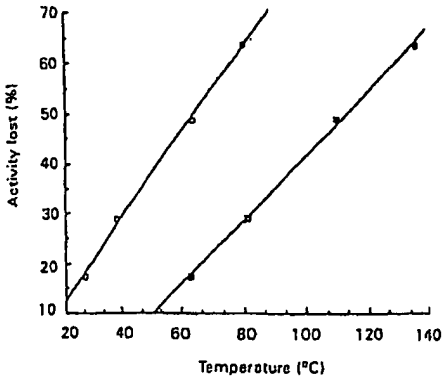

Fig. 1. The effect of drier inlet and outlet temperature on the residual activity of β-galactosidase spray-dried without protective excipients. ■ Inlet temperature, ○ outlet temperature. (Solution feed rate, 3·5 mL min^{-1}, air flow rate, 600 L h^{-1}.)

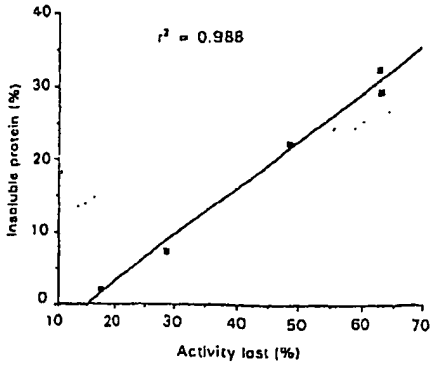

Fig. 2. The relationship between activity loss and the proportion of insoluble protein in β-galactosidase samples spray-dried without protective excipients.

Effect of process variables

Table 3 shows the fitted equations relating the response

Table 3. Fitted equations relating product characteristics to processing variables.

Drier outlet temperature (°C)	$Y_1 = 59{\cdot}2 + 21{\cdot}1X_1 - 8{\cdot}7X_2 - 1{\cdot}8X_4$	$r^2 = 0{\cdot}995$
Residual activity (%)	$Y_2 = 76{\cdot}6 - 22{\cdot}3X_1 + 12{\cdot}6X_2 + 11{\cdot}6(X_1 \cdot X_2)$	$r^2 = 0{\cdot}979$
Geometric mean particle size (μm)	$Y_3 = 4{\cdot}07 + 0{\cdot}41X_1 + 0{\cdot}30(X_2 \cdot X_3) - 0{\cdot}20(X_1 \cdot X_2)^* + 0{\cdot}39X_4$	$r^2 = 0{\cdot}926$
Moisture content (%)	$Y_4 = 5{\cdot}30 - 0{\cdot}20X_1 - 0{\cdot}22X_2 + 0{\cdot}23X_3 - 0{\cdot}29(X_1 \cdot X_2) + 0{\cdot}27(X_2 \cdot X_3) - 0{\cdot}16X_4{}^*$	$r^2 = 0{\cdot}883$
Yield (%)	$Y_5 = 15{\cdot}6 + 3{\cdot}9X_1 - 7{\cdot}5X_2 - 6{\cdot}9(X_1 \cdot X_2) + 4(X_2 \cdot X_3)$	$r^2 = 0{\cdot}923$

* Significant at 90% confidence level. X_1: Inlet temperature; X_2: feed rate; X_3: air flow rate; X_4: day.

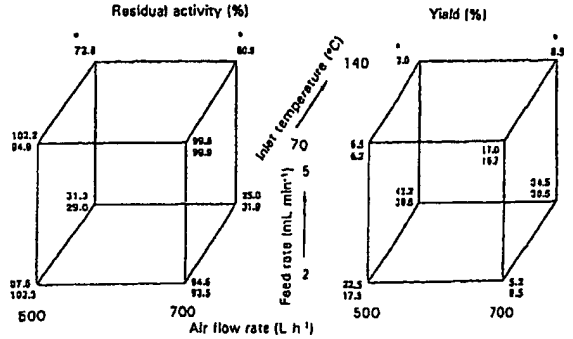

Fig. 3. The effect of process variables on residual activity and yield. *Indicates missing data points where yields were too small to permit analyses to be carried out.

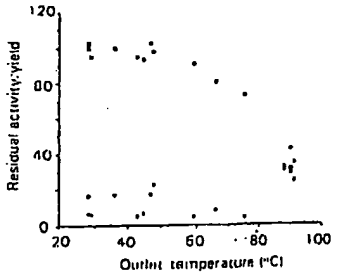

Fig. 4. The effect of drier outlet temperature on residual activity and yield. ● Yield, ■ residual activity.

Table 4. The effect of formulation additives on the properties of spray-dried β-galactosidase.

Stabilizer	Moisture content (%)	Geometric mean particle size	Residual activity following drying (%)
None	4·5	3·9	42
Mannitol	1·5	4·3	81
Sucrose	2·8	4·3	102
Arginine HCl	3·2	4·4	106
Trehalose	4·0	4·0	109

parameters to the processing factors. Terms included in the equations were significant at the 95% level unless otherwise marked.

Several terms were found to have statistically significant effects on the product moisture content. However, the magnitude of the effects was small and of little practical importance. Labrude et al (1989) reported a decrease in moisture content with an increase in drying temperature when a haemoglobin and sucrose formulation was dried using a Büchi spray-drier. It has been reported that the moisture content of the product is determined by the outlet temperature of the spray-drier (Masters 1985); this effect was not observed in this study over a temperature range of 70–140°C.

A tendency for the particle size to increase with increasing drier inlet temperature was observed. This effect is probably due, at least in part, to the increased tendency to agglomerate exhibited by the powders spray-dried at high temperatures. The effect of temperature on particle size is reported to be dependent on the material being dried (Crosby & Marshall 1958), and so this observation is probably formulation specific. In two-fluid driers such as the Büchi 190, the particle size is reported to decrease with an increase in the ratio of the air to liquid flow rates, since this represents an increase in the energy available for atomization (Masters 1985). Our model supports this theory since when both flow rates are high or low (minimizing this ratio), the $X_2 \cdot X_3$ interaction term is

Table 5. The effect of storage on the residual activity of spray-dried β-galactosidase formulations.

Conditions	Residual activity				
	No stabilizer	Mannitol	Sucrose	Arginine HCl	Trehalose
Initial	41·8	80·5	101·5	105·8	108·6
5°C					
1 month	30·0	62·3	106·2	99·9	101·8
6 months	27·4	43·2	95·1	97·8	100·7
12 months	19·9	24·7	95·9	94·7	96·0
30°C					
1 week	26·5	50·7	103·2	96·9	109·3
1 month	21·2	33·0	100·5	104·5	110·3
4 months	17·6	28·7	100·3	104·6	100·4
6 months	18·0	26·7	90·2	91·8	95·9
12 months	11·7	20·5	88·6	97·5	96·8
40°C					
1 week	24·8	35·1	101·0	105·2	108·6
1 month	19·6	19·2	102·2	109·8	106·6
4 months	15·1	18·5	87·6	105·9	104·7
6 months	13·3	16·5	81·4	96·2	99·7
12 months	9·2	13·3	74·1	95·5	107·4

positive and hence results in an increase in particle size. Although this model fits the data quite well, the day-to-day variability (X_4) is significant, and is larger than any change which could be induced by alteration of process variables. Since the day-to-day variation is confounded with the three factor interaction effect ($X_1 \cdot X_2 \cdot X_3$) however, it is impossible to differentiate the relative importance of their respective contributions.

The outlet temperature of the drier was determined solely by the main effects of the inlet temperature and the solution feed rate. At a given inlet temperature, a decrease in feed rate will cause the outlet temperature to rise. The outlet temperature of the spray-drier is considered to be the most important factor in determining the residual activity of spray-dried heat sensitive materials (Duemen & van der Stege 1982; Labrude et al 1989). With the Büchi apparatus, the outlet temperature cannot be controlled directly, but the fitted equation shows that it can be predicted with considerable accuracy from a knowledge of the inlet temperature and the solution feed rate (Table 3, eqn 1). Similarly, it enables the processing conditions to be controlled so as to give a desired outlet temperature. The extent to which this relationship would change with different formulations is uncertain, but it is unlikely to be substantially altered as long as the viscosity of the solution remains low.

The fitted equation in Table 3 indicates that the residual activity is determined by the main effects of solution feed rate and drier inlet temperature with the interaction between the two also being significant. It can be seen from Fig. 3 that increasing the inlet temperature results in much greater activity losses when the feed rate is low, compared with when feed rate is high. This phenomenon is reflected in the $X_1 \cdot X_2$ interaction term in the fitted equation (Table 3, eqn 2). This can be explained by the manner in which inlet temperature and solution feed rate control outlet temperature, as described above. The relationship between residual activity and drier outlet temperature is illustrated in Fig. 4. It can be seen from the nature of the curve that the residual activity is much more sensitive to small changes in outlet temperature when the temperature is high, than at low or moderate temperatures.

The yield was also determined by the drier inlet temperature, the liquid feed rate and the interaction between the two. Here again the interaction term is of critical importance as illustrated in Fig. 3. An increase in inlet temperature improves yield only when the solution feed rate is low. At high solution feed rates yields are low, irrespective of drying temperature. The fitted equation confirms these observations; the yield can be considerably increased by reducing the solution feed rate. A simultaneous increase in the drier inlet temperature results in dramatic improvements in the product yield. The yield is therefore highly dependent on drier outlet temperature as shown in Fig. 4. A positive value of the $X_2 \cdot X_3$ term (i.e. a low airflow:feed rate ratio) further increases the yield. Since a low feed rate is required to obtain high product yields, it follows that a low air flow rate is also required to maximize the yield.

The strong interaction effects observed between inlet temperature and solution feed rate, with respect to residual activity and yield, highlight the value of using statistically designed experiments. Had the effects of the process variables been studied individually, varying one factor at a time, these findings would have been missed.

It is evident from Figs 3 and 4 that the highest product yields are associated with significant losses in enzymatic activity. The experimental region where yields are greatest corresponds to the region where residual activity levels are lowest. Clearly a prerequisite for the use of this process would be that drying could be accomplished without any activity losses. Nevertheless low yields are a persistent problem with laboratory-scale spray-driers, and may place severe restrictions on the use of the process in early product development of protein drugs. Thus, it is obviously desirable to be able to carry out the process under conditions which maximize yields. This led us to investigate potential stabilizers which might be effective in stabilizing the enzyme under the harsher drying conditions required to achieve acceptable yields.

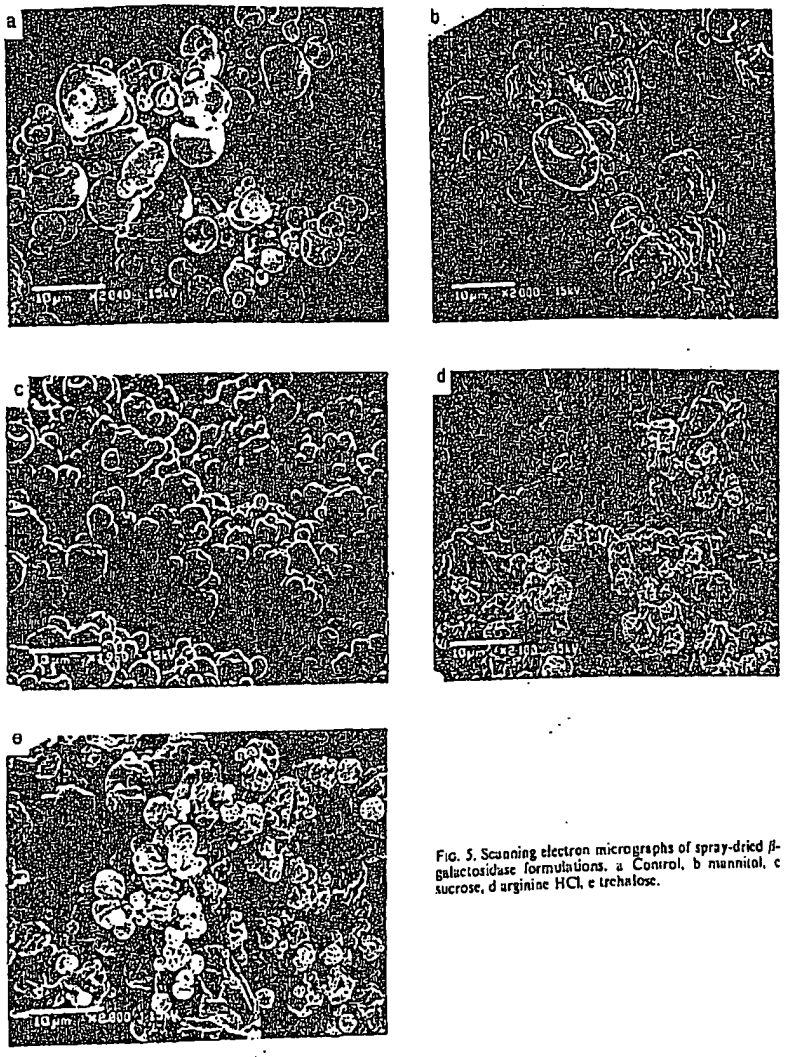

FIG. 5. Scanning electron micrographs of spray-dried β-galactosidase formulations. a Control, b mannitol, c sucrose, d arginine HCl, e trehalose.

Table 6. Fitted equations relating product characteristics to formulation variables.

Yield (%)	$Y_1 = 48{\cdot}8 - 10{\cdot}9X_1 + 7{\cdot}1X_3 - 5{\cdot}3X_4 - 5{\cdot}0(X_4 \cdot X_5)$*	$r^2 = 0{\cdot}74$
Residual activity (%)	$Y_2 = 78{\cdot}5 + 12{\cdot}0X_2 - 39{\cdot}3X_3 + 7{\cdot}4(X_2 \cdot X_3)$*	$r^2 = 0{\cdot}91$
Residual moisture content (%)	$Y_3 = -0{\cdot}05^{+} - 0{\cdot}26X_1^{+} - 0{\cdot}30X_2 - 0{\cdot}44X_4 - 0{\cdot}77X_5 + 0{\cdot}36(X_2 \cdot X_4) - 0{\cdot}36(X_3 \cdot X_5) + 0{\cdot}40(X_4 \cdot X_5)$	$r^2 = 0{\cdot}82$
Geometric size (μm)	$Y_4 = 2{\cdot}09 - 0{\cdot}43X_1 + 0{\cdot}72X_4 - 0{\cdot}74X_5 + 0{\cdot}05$r.h. $+ 0{\cdot}28(X_1 \cdot X_2)$	$r^2 = 0{\cdot}77$

* Significant at 90% confidence level. + Not significant. X_1: Surfactant; X_2: stabilizer (+trehalose, −arginine HCl); X_3: total solids; X_4: stabilizer:enzyme ratio; X_5: inlet temperature; X_4, X_5: day.

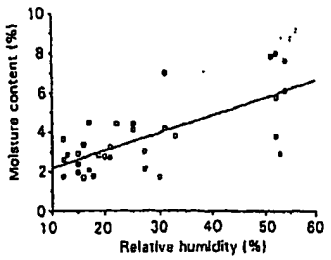

FIG. 6. The effect of ambient relative humidity on the moisture content of spray-dried formulations.

Effect of excipient

Table 4 shows the properties of formulations containing 6% β-galactosidase and 10% arginine hydrochloride, sucrose, trehalose or mannitol in deionized water. These formulations were dried at a feed rate of 2 mL min^{-1}, an air flow rate of 500 L h^{-1} and at an inlet temperature of 105°C. Yields of 40–65% were obtained. Table 5 shows the effect of storage at 5, 30 and 40°C on the residual activity of the product. The control and mannitol formulations which both suffered activity losses during the spray-drying process continued to degrade during storage. As would be expected, the activity losses progressed faster at higher storage temperatures. Whilst mannitol evidently has a slight protectant effect compared with the control formulation, the other stabilizers are all more effective. Arginine hydrochloride and trehalose were able to maintain the potency of β-galactosidase both during spray-drying and during storage at 40°C for one year. Both these substances have previously been reported to stabilize *A. oryzae*-derived β-galactosidase during freeze-drying and subsequent storage at 70°C for a period of seven days (Izutsu et al 1991). The sucrose formulation was stable for one year at 5°C, but started to lose activity after about three months storage at higher temperatures. This formulation also developed a brown discoloration after prolonged storage at the higher temperatures. This can be attributed to Maillard reactions which occur when sucrose is hydrolysed to its constituent monosaccharides (Roser 1991; te Booy et al 1992). This phenomenon has been shown to occur in freeze-dried protein preparations containing sucrose even when prepared under neutral or only mildly acidic conditions (Tarelli & White 1982). Those authors also observed that mannitol and trehalose possessed much greater inherent stability than sucrose and lactose, especially when the product moisture content was greater than 1%. It is interesting to note that the excipients which provided the greatest degree of protection in the spray-drying process also afforded the best long-term stability. This may indicate a similarity in the mechanism of protection from the two different types of thermal stress.

Fig. 5 shows scanning electron micrographs of the formulations described above. There is a striking difference between the smooth-surfaced particles observed in the control formulation containing no excipient, and the more pitted surfaces observed when stabilizers were included in the formulation. This is particularly apparent in the case of the arginine hydrochloride and trehalose formulations. A small increase in particle size (1–2 μm) was observed for the sucrose and arginine hydrochloride samples after storage at 30 and 40°C. This occurred because these samples tended to form hard cakes during storage which did not easily disperse during the slide preparation process. Therefore, the apparent increase in size can be attributed to the presence of powder agglomerates on the slides. This effect was observed to a lesser extent with the control and mannitol formulations, and did not occur at all with the trehalose formulation, which maintained its fine, powdery appearance even after twelve months storage at 40°C.

Effect of formulation variables

From the results observed in the stability studies, arginine hydrochloride and trehalose were judged to be the most promising stabilizers and were therefore investigated further.

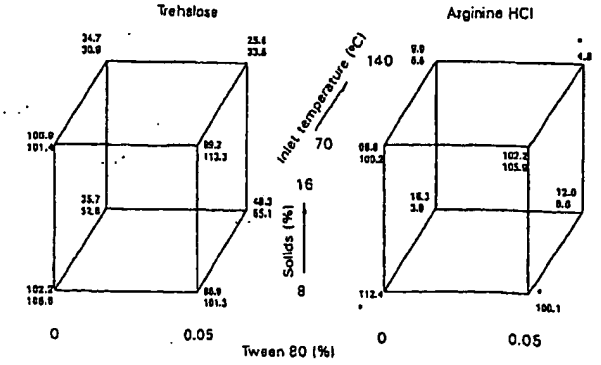

FIG. 7. The effect of total solids, presence of surfactant and drying temperature on the residual activity of arginine hydrochloride and trehalose formulations. * Indicates missing data points where yield was too small to permit analyses to be carried out.

A 2^3 full factorial experimental design was used to compare these two stabilizers, and also to evaluate the effect of total solids (8–16%), stabilizer to protein concentration ratio (1·7:1–7:1), presence of surfactant (0·05% Tween 80) and drying temperature (105–170°C). Drying temperature was included in this experimental design since we wished to evaluate the effect of higher drying temperatures than those used in the first phase of the study. A feed rate of 2 mL min^{-1} and an air flow rate of 500 L h^{-1} were used throughout this study. The 32 experimental runs were blocked into four sets of eight runs. Again the three-factor interactions were confounded with the day-to-day variation. Table 6 shows the fitted equations obtained from the experimental data.

There was substantial variation in the ambient relative humidity on the four occasions on which the spray-drying was conducted. Levels ranged from less than 15% to over 50%. These variations clearly affected the moisture content of the product. In addition, the yields from the arginine hydrochloride runs carried out when the relative humidity was high were very poor, since the product obtained was extremely sticky and could not easily be removed from the product collector. The effect of relative humidity on the product moisture content is illustrated in Fig. 6. Although the r^2 value for this fitted equation is only 0·506, it must be remembered that this plot shows data from all the experimental runs and so was not obtained under constant conditions with respect to the five experimentally controlled parameters. Statistical analysis of the data showed a highly significant effect of relative humidity on product moisture content ($P=0·0001$). Thus the residuals procedure of SAS was used to eliminate the effect of relative humidity from the data, so that the effects of the experimentally controlled variables could be analysed. Using this procedure, the portion of the moisture content which is due to the ambient humidity is subtracted from the experimental data to give the residual moisture content. The residual data values can then be analysed in the usual manner to test for the effect of the experimentally controlled variables (Daniel & Wood 1980).

Comparison of arginine hydrochloride and trehalose formulations

Fig. 7 illustrates the effect of solids, surfactant and drying temperature on the residual activity of arginine hydrochloride and trehalose formulations. This figure clearly shows a greater ability of trehalose to preserve the enzymatic activity at higher drying temperatures. This was confirmed by a subsequent experiment in which trehalose and arginine hydrochloride formulations were dried at inlet temperatures between 105 and 200°C (Fig. 8). Data obtained when β-galactosidase was spray-dried without additives were included in this plot for comparison. The ability of trehalose to stabilize proteins is currently the subject of much debate. It has been reported to have remarkable stabilizing properties in the air-drying of various biologically active materials (Roser 1991; Colaço et al 1992), although other researchers have found sucrose to be equally effective (Levine & Slade 1992). Nevertheless, trehalose appears to be extremely effective in preventing thermal denaturation of β-galactosidase during spray-drying, and allows drying to be carried out at a temperature at least 20°C higher than is possible when either sucrose or arginine hydrochloride is used as the stabilizer.

The regression equations in Table 6 also show a slightly smaller particle size and a slightly lower moisture content when trehalose is used as a stabilizer rather than arginine hydrochloride. Trehalose was therefore selected as the stabilizer of choice for spray-drying β-galactosidase.

Determination of final formulation and process for trehalose formulation

Table 7 shows the regression equations obtained using only the data from the trehalose runs. This procedure results in simpler regression equations, containing fewer terms since the stabilizer term and its respective interaction terms have been removed.

Both the product moisture content and residual activity were only affected to a statistically significant extent by the drying temperature. An increase in drying temperature caused a reduction in both residual activity and moisture content. It is interesting that in these experiments there was a clear effect of temperature on the product moisture content, since this effect was expected, but not observed, in the

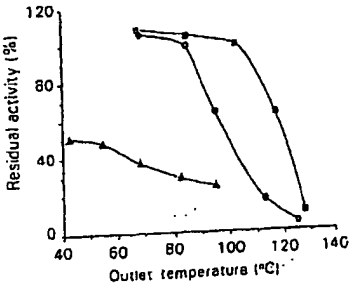

FIG. 8. The effect of spray-drier outlet temperature on the residual activity of arginine hydrochloride (●), trehalose (■), and control (▲) formulations. Pump speed, 2 mL min^{-1}; air flow rate, 500 L h^{-1}.

Table 7. Fitted equations relating product characteristics of the trehalose formulation to the formulation variables.

Yield (%)	$Y_1=33·8-11·0X_1+9·5X_3-4·2(X_1\cdot X_4)^*$	$r^2=0·92$
Residual activity (%)	$Y_2=89·5-31·9X_3$	$r^2=0·81$
Residual moisture content (%)	$Y_3=0·03^*-1·15X_3+0·16(X_4\cdot X_5)^*$	$r^2=0·87$
Geometric mean size (μm)	$Y_4=2·62-0·35X_4-0·50X_5^*$	$r^2=0·81$

* Significant at 90% confidence level. * Not significant. X_1: Surfactant; X_3: total solids; X_4: stabilizer:enzyme ratio; X_5: inlet temperature; $X_4.X_5$: day.

previous experiments which focused on processing variables. It may be because the temperature range in the initial study was only 70–140°C, whereas in this study a range of 105–175°C was used. In addition, the very low yields obtained in the earlier experiments may have led to misleading moisture content results.

Clearly there is no benefit to be gained by including Tween 80 in the formulation, since its only significant effect was to reduce the yield from the drier. Formulations containing Tween 80 tended to form small spherical beads in the drier which did not collect as efficiently as the fine powders from the other runs, and lower yields were observed. The yield could be increased by almost 20% by using a high (16%) rather than a low (8%) total solids content. It therefore seems sensible to use as high a total solids content as possible, since this also reduces the time required to dry a given amount of material.

The ratio of stabilizer to protein affected only the geometric mean size of the product. A slightly smaller mean size could be obtained by increasing the proportion of trehalose in the formulation. Any potential benefit resulting from this size decrease, however, would probably not be sufficient to merit the large increase in the amount of excipient.

To minimize the product moisture content, it is obviously desirable to operate at the maximum drying temperature which can be used without causing activity losses. Present data indicate that an inlet temperature of 140°C and an outlet temperature of 95–100°C can be used, provided that cooling water is circulated through the drier nozzle to prevent thermal denaturation in the nozzle.

Effect of feed solution pH

A final study was carried out to evaluate the effect of the pH of the feed solution on the residual activity of the trehalose formulation. Up to this point, all experimental runs had been carried out in a simple aqueous solution containing only the protein and a stabilizer (approx. pH 5·8–6·5). *A. oryzae*-derived β-galactosidase has a reported isoelectric point of about pH 4·5 as determined by isoelectric focusing (Ogushi et al 1980), and formulations of pH 2·5, 4·5 and 6·5 were evaluated so that any effect of the overall charge on the protein could be observed. The protein solutions were prepared in phosphate-citrate buffer adjusted to the desired pH and dialysed against the appropriate buffer for 24 h. The pH 2·5 formulation was not spray-dried, since degradation and precipitation of the protein occurred during the dialysis. Table 8 shows the residual activity of the pH 4·5 and 6·5 formulations spray-dried at inlet temperatures of 140 and 150°C. Two different buffer strengths were evaluated. The pH 4·5 formulation degraded to a much greater extent than the pH 6·5 formulation, which behaved in the same manner as the unbuffered formulation. The buffer strength also affected the residual activity of the pH 4·5 formulation, with less activity loss occurring at both temperatures when the ionic strength was reduced. Since the pH 6·5 buffered solution behaved in the same fashion as the unbuffered solution, a buffer was not included in the formulation.

The final formulation and process is shown in Table 9. Drying under these conditions causes no activity loss, and yields of over 70% have been achieved. The geometric mean size of the product is typically between 3 and 4 μm, an ideal size for inclusion in dry-powder inhaler devices. The particle size and size distribution were not greatly affected by any of the process or formulation variables, and are probably largely a function of the spray-drier design. It is likely that to effect any significant changes in particle size, a different nozzle would be required.

Table 8. The effect of feed solution pH and ionic strength on the residual activity of spray-dried β-galactosidase.

Feed solution formulation (phosphate/citrate)	Residual activity (%)	
	140°C	150°C
pH 4·5 (0·2 M/0·1 M)	69·8	42·0
pH 4·5 (0·1 M/0·05 M)	87·5	60·5
pH 6·5 (0·2 M/0·1 M)	98·9	81·8
pH 6·5 (0·1 M/0·05 M)	98·5	79·0
pH 6·0 (unbuffered)	101·6	71·6

Table 9. Optimal formulation and process.

Stabilizer	10% Trehalose
Solids	16%
Stabilizer:protein ratio	1·67:1
Other excipients	None
Inlet temperature	140°C
Outlet temperature	95–100 C
Solution feed rate	2 mL min^{-1}
Air flow rate	500 L h^{-1}

The moisture content of the final formulation is typically between 2 and 5%. This study clearly demonstrated the effect of ambient relative humidity on the moisture content of the product, and it would be desirable to carry out the spray-drying process in a low humidity environment. Under these conditions moisture levels of around 2–3% could be achieved.

This study indicates that spray-drying is a valuable technique in the formulation of protein drugs. By judicious control of processing variables, yields of fully active protein in excess of 70% are achievable. The identification of an efficient stabilizer is likely to be of paramount importance in the development of a spray-dried formulation of any protein drug. A relatively high aqueous solubility is advantageous, since spray-drying operates best when the feed solution has a high solids content. Furthermore, the difficulties encountered in obtaining adequate yields from laboratory-scale spray-driers may be resolved during the scaling-up process. Spray-drying thus merits consideration by protein formulators when the control of particle size is necessary or desirable.

Acknowledgements

The authors would like to thank the United States Pharmacopeial Convention and SmithKline Beecham Pharmaceuticals for financial support provided to J. Broadhead. We would also like to thank P. Schweibenz for preparing the electron micrographs and Drs T. Sokoloski and J. Baldoni for the helpful discussions in the preparation of the manuscript.

References

Box, G. E. P., Hunter, W. G., Hunter, J. S. (1978) Statistics for Experimenters. An Introduction to Design, Data Analysis and Model Building. Wiley, New York

Broadhead, J., Edmond Rouan, S. K., Rhodes, C. T. (1992) The spray drying of pharmaceuticals. Drug Dev. Ind. Pharm. 18: 1169–1206

Cady, S. M., Steber, W. D., Fishbein, R. (1989) Development of a sustained release delivery system for bovine somatotropin. Proceedings of the International Symposium on Controlled Release and Bioactive Materials 16: 22–23

Colaço, C., Sen, S., Thangavelu, M., Pinder, S., Roser, B. (1992) Extraordinary stability of enzymes dried in trehalose: simplified molecular biology. Bio/Technology 10: 1007–1011

Crosby, E. J., Marshall, W. R. (1958) Effects of drying conditions on the properties of spray dried particles. Chem. Eng. Prog. 54: 56–63

Daemen, A. L. H., van der Stege, H. J. (1982) The destruction of enzymes and bacteria during the spray drying of milk and whey. 2. The effect of the drying conditions. Neth. Milk Dairy J. 36: 211–229

Daniel, C., Wood, F. S. (1980) Fitting Equations to Data. 2nd edn, Wiley, New York

Food and Chemical Codex (1981) 3rd edn, National Academy Press, Washington D.C., p. 491

Izutsu, K., Yoshioka, S., Takeda, Y. (1991) The effects of additives on the stability of freeze-dried β-galactosidase stored at elevated temperature. Int. J. Pharm. 71: 137–146

Johnson, C. A. (1967) The Karl Fischer method. Adv. Pharm Sci. 2: 251–267

Labrude, P., Rasolomanana, M., Vigneron, C., Thirion, C., Chaillot, B. (1989) Protective effect of sucrose on spray drying of oxyhemoglobin. J. Pharm. Sci. 78: 223–229

Leving, H., Slade, L. (1992) Another view of trehalose for drying and stabilizing biological materials. Biopharm. 5: 4: 36–40

Masters, K. (1985) The Spray Drying Handbook. 4th edn, Longman, London

Masters, K. (1990) Understanding and applying spray dryers in chemical processing. Powder Bulk Eng. April: 36–44

Mumenthaler, M., Hsu, C., Pearlman, R. (1990) Preparation of protein pharmaceuticals using a spray drying technique. Pharm. Res. 7: S59

Ogushi, S., Yoshimotol, T., Tsuru, D. (1980) Purification of comparison of two types of β-galactosidase from *Aspergillus oryzae*. J. Ferment. Technol. 58: 115–122

Roser, B. (1991) Trehalose drying: a novel replacement for freeze-drying. Biopharm. 4: 8: 47–53

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. Anal. Biochem. 150: 76–85

Stubbs, W., Gibbons, E., Cady, S. M. (1988) Compositions for parenteral administration [illegible] European Patent Application [illegible]

Tanaka, Y., Kagamiishi, A., Kiuchi, A., Horiuchi, T. (1975) Purification and properties of β-galactosidase from *Aspergillus oryzae*. J. Biochem. 77: 241–247

Tarelli, E., White, C. A. (1982) Additives to biological substances II. Relative stabilities of some carbohydrate additives at and around neutral pH values in phosphate buffers and water. J. Biol. Stand. 10: 95–103

te Booy, M. P. W. M., de Ruiter, R. A., de Meere, A. L. J. (1992) Investigation of the physical stability of freeze-dried sucrose-containing formulations by differential scanning calorimetry. Pharm. Res. 9: 109–114

√: bestellt am 29.04.04